Sex discrimination of Buloke (*Allocasuarina luehmannii*) for selective revegetation

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Abstract

Allocasuarina luehmannii (Buloke) is a slow-growing dioecious species occurring primarily in south-eastern Australia. Larger seed-bearing female trees, at least 100 yrs old, are an essential food source for the threatened *Calyptorhynchus banksii graptogyne* (Karak). Sex-discrimination in Buloke was investigated utilising Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) techniques. While described as a dioecious species, 18% of the plants sampled were monoecious suggesting that this is in fact a subdioecious species. Stem material from 55 paddock trees was collected to identify genetic differences between female, male and monoecious plants. An initial screening of 56 primers, on a subset of five female, five male and five monoecious samples, identified 21 RAPD and 21 ISSR primers with clear scorable bands, but no unique female bands were amplified. Investigation into the sex determination mechanism of this species and the possible effect of environmental factors on sex expression is recommended before further sex-specific marker research is undertaken.

Keywords: sex-linked DNA markers, ISSR and RAPD analysis, subdioecy

Muelleria 29(2): 104-109 (2011)

Introduction

Buloke (*Allocasuarina luehmannii* (R.T.Baker) L.A.S.Johnson) woodland occurs in grassy open forest areas in south-eastern Australia (Department of the Environment, Water, Heritage and the Arts 2010). Vast tracts of this endangered vegetation community have been cleared for agriculture with only 3% of the original cover remaining (EPBC 1999). The endangered Karak or Red-tailed black cockatoo (*Calyptorhynchus banksii graptogyne*) feeds on mature cones of remnant Buloke for a short period each year when its staple food source is not available (Maron & Lill 2004). This has serious implications for the Karak as food availability has been suggested as a factor limiting population size (Maron & Lill 2004). Recently, farmers have set aside profit-making land for the revegetation of Buloke woodland to aid in the protection of the Karak. The propagation and revegetation of this species as a future food source for the Karak presents a number of challenges including an undetermined sex-expression mechanism and difficulty in *ex-situ* propagation.

Buloke is described as a dioecious species (Walsh & Entwistle 1996), so only a proportion of mature trees represent a potential food source. Revegetation with a higher proportion of female trees would concentrate the food source for the Karak in a smaller land area, thus meeting primary production and conservation needs. Females do not reach sexual maturity for ten to fifteen years and thus sexing seed propagated plants before field planting is not practical. The cone-bearing females do not reach a size suitable for Karak feeding for almost 100 years (Maron 2005). Clonal propagation from heavily seed bearing female trees is a possible tool for ensuring higher proportion of female trees in revegetation programs. Cutting propagation has been successful in other species of Casuarinaceae (Kondas 1981). Stem cuttings from the canopies of eight mature female Bulokes failed to strike, although stems harvested from a sucker propagated successfully (Delpratt, Conomikes and Wright, unpub. data). While promising, this solution would result in lower levels of genetic diversity in Buloke plantings in comparison to raising plants from seed. Populations consisting of clonal material from a limited number of parent plants could have decreased fitness levels and greater susceptibility to pests and diseases. Often, this does not

become apparent for several generations, which would be especially problematic in a slow-growing species like Buloke.

The development of a simple cost effective genetic test to distinguish female individuals would allow seedlings to be used as the primary propagule for revegetation with high proportions of females, and thus enhance the genetic diversity in the plantings. There have been a number of studies developing such tests in economically important dioecious plants such as *Salix viminalis* L. (Common Osier) (Alstrom-Rapaport *et al.* 1998), *Carica papaya* L. (Gangopadhyay *et al.* 2007), *Trichosanthes dioica* Roxb. (Pointed Gourd) (Kumar *et al.* 2008) and *Phoenix dactylifera* L. (Date Palm) (Younis *et al.* 2008). The majority of these studies have utilised Inter Simple Sequence Repeat (ISSR) or Random Amplified Polymorphic DNA (RAPD) analysis and occasionally Amplified Fragment Length Polymorphism (AFLP) analysis to identify consistent genetic differences between females and males (and in some cases hermaphrodites). Once consistent differences have been identified by RAPD, ISSR or AFLP, it is possible to convert the amplification products to a simple PCR-based test called Sequence Characterised Amplified Regions (SCAR) to discriminate female plants at the seedling stage (Gunter *et al.* 2003). The use of SCAR markers in plant conservation represents a novel application of this technique.

Interestingly, there have been reports of monoecious trees in small proportions of Wimmera Buloke populations (pers. comm. M. Maron 2009). The presence of both dioecious and monoecious plants in a population, known as subdioecy, is believed to be one of the intermediate sexual systems on the evolution from hermaphroditism (or monoecy) to dioecy (Charlesworth *et al.* 2005; Ming *et al.* 2007). While it is still possible to identify sex-linked genetic differences in subdioecious systems (Chaves-Bedoya & Nuñez 2007), these reports need to be investigated so that all sexual morphs can be included in the initial screen for genetic differences.

The aim of this study was to identify consistent genetic differences between male, female and monecious Buloke plants to allow the female individuals to be distinguished prior to reproductive maturity. To achieve this we began by investigating reports of occasional monoecy in Wimmera populations of Buloke. Secondly, we used RAPD and ISSR techniques to screen for consistent genetic differences between male, female and monoecious plants.

Methods

In December 2008, paddock trees near Edenhope, Victoria (37°3'S, 141°20'E) were investigated to estimate gender ratios and collect stem material for DNA extraction. The gender of 55 trees was distinguished by the presence of staminate flowers (Fig. 1a) for

Figure 1. Morphological characteristics used to assign gender to Buloke plants. 1*a* staminate flowers and 1*b* female cones.

males, seed bearing cones (Fig. 1b) for females or both for monoecious plants. Herbarium specimens were prepared for lodgement in the National Herbarium of Victoria (MEL). In preparation for DNA extraction stems from each plant sampled were stored at -20°C. Then 100 mg of each sample was cleaned with 0.5 mL/L Triton X, followed by a rinse with sterile distilled water, and emersion in 1 mL/L of 20% SDS rinsed with sterile distilled water then crushed to a powder in a mortar and pestle using liquid nitrogen. Qiagen® DNeasy mini kits were used to isolate DNA from each sample according to the manufacturer's instructions. Samples were run on a 1.5% agarose gel containing 1 µL /10 mL Sybrsafe at 110 V for 1.5 hours and visualised using UV light to determine DNA concentration. DNA concentration was standardised to 5 ng/µL before use in RAPD and ISSR reactions.

Each 25 µL RAPD reaction contained 10 ng of DNA, 12.5 µL of GoTaq® Green Master Mix, 0.8 mM of each primer and made to volume with sterile MilliQ® water. RAPD reactions were conducted using a Corbett Thermal Cycler with an initial strand separation cycle of 94°C for two minutes followed by 45 cycles of 94°C for 30 seconds, 34°C for 30 seconds, 72°C for one minute with a final extension of 72°C for five minutes. Five µL of each reaction was loaded on a 2% agarose gel and

Table 1. The ISSR and RAPD primers used in this research.

ISSR Primers	Sequence	RAPD Primers	Sequence
Primers that produce amplification products:			
807	AGA GAG AGA GAG AGA GT	OP81	GTT TCG CTC C
B09	AGA GAG AGA GAG AGA GG	OP820	GGA CCC TTA C
810	GAG AGA GAG AGA GAG AT	OPC11	AAA GCT GCG G
811	GAG AGA GAG AGA GAG AC	OPC16	CAC ACT CCA G
814	CTC TCT CTC TCT CTC TTG	OPD13	GGG GTG ACG A
822	TCT CTC TCT CTC TCT CA	OPH8	GAA ACA CCC C
823	TCT CTC TCT CTC TCT CC	OPQ9	GGC TAA CCG A
824	TCT CTC TCT CTC TCT CG	OPV1	TGA CGC ATG G
840	GAG AGA GAG AGA GAG AYT	OPV2	AGT CAC TCC C
841	GAG AGA GAG AGA GAG AYC	OPV3	CTC CCT GCA A
842	GAG AGA GAG AGA GAG AYG	OPV5	CCC CTC ACG A
844a	CTC TCT CTC TCT CTC TAC	OPV6	ACG CCC AGG T
849	GTG TGT GTG TGT GTG TYA	OPV7	GAA GCC AGC C
850	GTG TGT GTG TGT GTG TYC	OPV8	GGA CGG CGT T
851	GTG TGT GTG TGT GTG TYG	OPV10	GGA CCT GCT G
868	GAA GAA GAA GAA GAA GAA	OPV12	ACC CCC CAC T
8L1	GAG AGA GAG AGA GG	OPV14	AGA TCC CGC C
BL2	GTG TGT GTG TGT GG	OPV15	CAG TGC CGG T
BL3	GAG AGA GAG AGA CC	OPV16	ACA CCC CAC A
8L4	GTG TGT GTG TGT CC	OPV19	GGG TGT GCA G
BL5	CAC CAC CAC GC	OPV20	CAG CAT GGT C
Primers that did not produce amplification products:			
831	ATA TAT ATA TAT ATA TYA	OPA4	AAT CGG GCT G
832	ATA TAT ATA TAT ATA TYC	OPC13	AAG CCT CGT C
833	ATA TAT ATA TAT ATA TYG	OPE16	GGT GAC TGT G
(GA)8N	GAG AGA GAG AGA GAG AN	OPV4	CCC CTC ACG A
(GT)8N	GTG TGT GTG TGT GTG TN	OPV9	TGT ACC CGT C
		OPV11	CTC GAC AGA G
		OPV13	ACC CCC TGA A
		OPV17	ACC GGC TTG T
		OPV1B	TGG TGG CGT T

electrophoresis was conducted for two hours at 110 V. Amplified bands were photographed with a Kodak Digital Science DC120 digital camera and size of bands by molecular weight was determined using a Gene Ruler® 100 base pair (bp) ladder as a reference (MBI Fermentas, Germany) with Kodak Digital Imaging® software. ISSR reactions were conducted as for the RAPD analysis using the cycle: 94°C for three minutes followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for one minute with a final extension of 72°C for ten minutes.

An initial screening of 26 ISSR and 30 RAPD primers (Table 1) was conducted using a single female DNA sample. The initial screening primers were selected from those shown to be successful for *Allocasuarina* species (Yasodha *et al.* 2004) and in the sex discrimination of other dioecious plants including *Salix viminalis* (Alstrom-Rapaport *et al.* 1998), *Carica papaya* (Gangopadhyay *et al.* 2007), *Trichosanthes dioica* (Kumar *et al.* 2008) and *Phoenix dactylifera* (Younis *et al.* 2008). Primers that produced clear bands in the initial screen were used to test for consistent genetic differences between five female, five male and five monoecious plants from site one. The ISSR primer BL4 was run with an extended sample set of 18 female, 18 male and 4 monoecious samples.

Results

Among the trees sampled were 23 female (42%), 22 male (40%) and 10 monoecious (18%) individuals. The initial screening of 56 primers resulted in 21 RAPD and 21 ISSR primers with clearly amplified bright bands (Table 1). The selected 21 RAPD and 21 ISSR primers were run with DNA from five male, five female and five monoecious plants. The ISSR analysis produced a total of 1044 bands ranging from 100 bp to 1200 bp in size, 474 of which were polymorphic (45%). The RAPD analysis produced 284 bands from 100 bp to 1500 bp in size, 209 of which were polymorphic (74%). In all cases where primers produced amplification products, bands were produced in at least one of each of the female, male and monoecious samples. There were no instances where bands were amplified in only one sex and none of the primers amplified a reproducible band unique to the female samples. The ISSR primer BL4 showed some promise, as it produced faint bands unique to three of five female samples in the first screening. However, when ISSR reactions were run with primer BL4 and an expanded DNA sample set from female (18), male (18) and monoecious (4) plants, no unique female markers were generated.

Discussion

Though *Allocasuarina luehmannii* is documented as a dioecious species (Walsh & Entwistle 1996) 18% of the samples collected had evidence of both staminate stems and female cones. This is an unusually high proportion of monoecious individuals in a population of dioecious species from the Casuarinaceae, with 2-3% monoecious individuals reported in *Casuarina equistifolia* L. (Yasodha *et al.* 2004) and less than 1% previously observed in nearby Wimmera *A. luehmannii* populations (pers. comm. Martine Maron 2010). The presence of monoecious plants in *A. luehmannii* populations suggests that this species is subdioecious rather than dioecious. Studies of the reproductive viability of the monoecious individuals are required to confirm this characterisation, as it is still unknown whether these plants produce both viable pollen and ovules. Comparing the amount of seed produced and cone size of monoecious and female plants, alongside investigations of feeding preferences between these sexual morphs, would help elucidate whether these plants represent a significant food source for the Karak.

Dioecy is relatively rare among flowering plants (Yampolsky & Yampolsky 1922; Renner & Ricklefs 1995). It is widespread across different lineages and is therefore believed to have evolved repeatedly, although usually within a single species (Westergaard 1958; Meagher 2007). However, dioecy is prevalent in some families such as the Salicaceae, Euphorbiaceae, Cucurbitaceae and the Casuarinaceae. The genetic basis of sex determination in flowering plants is variable (Irish & Nelson 1989), with genetic mechanisms ranging from a single locus on an autosomal chromosome to the presence of heteromorphic X and Y chromosomes containing multiple genes involved in sex determination (Ming *et al.* 2007). A higher proportion of sex-specific markers are expected in the presence of X and Y chromosomes as, due to the difference in chromosomes between males and females, there are a larger number of sex-specific genes in this system. The genetic mechanism for sex determination in the Casuarinaceae

is still unknown. The Casuarinaceae originated outside of Australia and non-Australian species have retained a fairly stable diploid chromosome number (Barlow 1959). Those species endemic to Australia have highly variable, often polyploid chromosome numbers (Barlow 1959). *Allocasuarina luehmannii* is one of the few tetraploid species ($2n = 56$) in the family.

No sex-specific markers were identified in *A. luehmannii* by this study (26 ISSR and 30 RAPD primers) or in *Casuarina equistifolia* by Yasodha *et al.* (2004) who screened 7 ISSR primers on DNA from 12 individuals. With the presence of X and Y sex chromosomes a relatively high number of sex-specific markers would be expected using ISSR and RAPD methods. This suggests that sex determination in Buloke, and in other members of the Casuarinaceae, is not due to the presence of X and Y sex chromosomes.

Given the variability of the genetic basis for sex determination in flowering plants it is not surprising that studies that have successfully identified sex-specific markers have required widely differing numbers of primers to successfully identify consistent differences between males and female plants; *e.g.* 15 RAPD for *Cannabis sativa* L., a dioecious species that has X and Y sex chromosomes (Sakatmoto *et al.* 1995) and 1,000 RAPD primers for *Salix viminalis* that appears to lack X and Y chromosomes (Gunter *et al.* 2003). It is possible that screening more primers than those used in the current study (*e.g.* >1,000) may identify sex-specific markers in Buloke, but other molecular methods may prove to be more useful. One such method, AFLP analysis, has been shown to produced a higher number of polymorphic bands per primer when compared to RAPD analysis (*i.e.* Sharma *et al.* 1996), thus increasing the likelihood of identifying a sex-specific markers.

In some plant species such as cucumber and maize sex expression is influenced by environmental factors such as light intensity, day length, temperature, mineral nutrition and plant hormones (Frankel & Galun 1977; Tandudzic & Banks 2004). It is important to establish whether or not one of these regulatory signals operates in the sex determination of Buloke plants. The identification of sex-specific markers may not give an accurate diagnosis of plant sex when external influences are involved, especially if plants are exposed to artificial growth environments such as nursery containers and glasshouses. Monitoring of sex ratios in multiple geographically widespread Buloke populations over a number of seasons may help to reveal whether changes in gender do occur. More direct studies exposing reproductively mature male, female and monoecious plants to different environmental conditions and plant hormone treatments under controlled conditions would help to elucidate what, if any, environmental factors influence sex expression in Buloke.

Further research into the sex determination mechanism in Buloke is recommended as it would help to predict the research effort required to locate sex-specific markers. In some sex determination systems such as XX:XO, where sex expression is influenced by dosage of the X chromosome rather than different genetic information (Hodgkin 2002), it is not possible to locate sex-specific markers. This system has not yet been described in plants. As there is no information on sex determination in the Casuarinaceae it is not possible that such a system could be responsible.

The aim of this project was to find genetic markers that can consistently identify female plants prior to fruiting. The use of RAPD and ISSR methods for this purpose relies on randomly locating sex-associated DNA regions. While the use of a wider range of RAPD and ISSR primers may achieve the above aim, it is unlikely that sex determination in Buloke is based on the presence of X and Y chromosomes, thus lacking the relatively large number of sex-specific regions of DNA that characterise this system. Therefore, we suggest that a much larger number of RAPD and ISSR primers and/or range of molecular methods is necessary in further studies to identify sex-specific markers in Buloke. It is also important to investigate the effect of environmental factors on sex expression and the genetic basis of sex determination in Buloke as these findings may make the identification of sex-specific markers redundant. In the mean time, the enhancement of revegetation plantings with high-bearing female trees will depend on refining clonal propagation techniques such as cutting propagation, micropropagation and the inducement of root suckers from selected paddock trees.

Acknowledgments

This study has been generously funded by the Kowree Tree Farm Group. We would also like to acknowledge the information from Martine Maron of The University of Queensland on sex ratios in Buloke paddock trees.

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